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Additive and synergistic effects of cyclosporine metabolites on glomerular mesangial cells

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Additive and synergistic effects of cyclosporine metabolites on glomerular mesangial cells. Out of the 29 cyclosporin (Cs) metabolites defined so far, 10 representative ones were isolated from bile of liver grafted patients, purified by HPLC, and their structure specified by FAB-MS and ^{1}H NMR. These were used to determine the growth inhibitory effects on Sprague Dawley rat glomerular mesangial cells (MC). Metabolite dilutions were added to cultured MC for 72 hours and [^{3}H]-thymidine incorporation was measured. A 50% growth inhibition by single metabolites (M) on MC was achieved at the following concentrations (mg/liter): Cs: 1.25; M21: 6.0; M18: 9.0; M26: 10.5; M1: 10.8; M8: 10.8; M17: 12.5; M13: >20.0; M25: >25.0; M203-218: >50.0; H355: >50.0. The activity was correlated to the degree of metabolization as the group of six "active" compounds included four primary metabolites (hydroxylated or demethylated derivatives of Cs: M21, M18, M1, M17), whereas the four "inactive" compounds exclusively were secondary metabolites (demethylated, hydroxylated and/or oxidized primary metabolites: M13, M25, M203-218, H355). Combinations of active metabolites with or without Cs resulted in an additive antiproliferative effect. Although single metabolite activities are not relevant in vivo, already combinations of three (M1 + M17 + M18) or four metabolites (M17 + M18 + M21 + H355) resulted in a significant growth inhibition at concentrations of the participating metabolites measured in urine of liver transplanted patients. Moreover, significant synergistic activities were determined with combinations including secondary metabolites. A final set of experiments discharged unspecific cytotoxic effects. The inhibition of MC [^{3}H]-thymidine incorporation was completely reversible and moreover, direct mesangiolysis was excluded for both single and combined metabolite actions. Thus, considering rat MC proliferation as an initial kidney cell model system for subsequent, more detailed studies measuring functional parameters, we have demonstrated that activities of single metabolites are related to their chemical structure. More importantly, mimicking to some extent the patients' situation, combinations of metabolites at concentrations occurring in vivo reduced MC proliferation in culture in an at least an additive fashion, suggesting that side effects of Cs treatment might be attributed to combined Cs metabolite actions.

The abundant use of the immunosuppressive drug cyclosporine (Cs) in transplantation and increasingly in the treatment of several autoimmune diseases [1–3] makes studies trying to elucidate the mechanisms of toxic effects important, especially as in the case of autoimmune disorders the applied daily doses are relatively high [2, 3]. In transplantation surgery the mean therapeutic doses have decreased in the recent years to reduce toxic effects without losing immunosuppressive efficacy [1, 2]. Despite close monitoring of Cs trough levels, almost 50% of the treated patients still suffer from serious renal [1–5] or neurological [4, 6] side effects of Cs therapy. There is increasing suspicion that Cs metabolites contribute to these undesirable complications [4, 6, 7].

To properly address the renal pathological alterations occurring during Cs treatment it is necessary to differentiate acute, functional Cs effects and irreversible kidney lesion after long-term therapy. Acute reduction of glomerular filtration rate (GFR) is a constant feature in all patients undergoing Cs therapy [1, 8, 9]. Although it is generally accepted that a primary vasoconstriction of afferent arterioles is associated with Cs application [1, 8, 9] there are sporadic investigations measuring a more pronounced increase in efferent arteriolar resistence [10]. The vasoconstrictive mechanisms underlying the Cs action are currently under discussion; a general sympathetic neural activation involving renal vessels was indicated by an earlier study in rats [11] and was recently supported by data from heart transplant patients [12]. Speculatively an influence of Cs on the gene expression of local humoral regulators of vasoactive compounds in endothelial or mesangial cells has been proposed [1]. However the most favored hypothesis, based on several experimental models [10, 13–17] and recent in vivo findings [18, 19], suggests a local imbalance of vasoactive substances, like platelet activating factor, prostaglandin E, prostacyclin and thromboxane, endothelin or angiotensin II. Cell culture experiments showing an enhancement of vasoconstrictor induced rise of intracellular calcium in MC or smooth muscle cells by Cs are in agreement with these investigations [20–23]. Unlike these reversible functional changes, during Cs therapy long-term treatment resulted in certain morphological lesions [2] in primarily unaffected kidneys [5]. These investigations revealed the following main alterations: tubulointerstitial damage, obliterative arteriolopathy, ischemic collapse (6 to 56%) or sclerosis of glomeruli (4 to 34%) associated with a significant enlargement of the remnant glomeruli and an expansion of the mesangial matrix. These toxic lesions, linked to Cs therapy, and finally leading to a decline in the number of functional glomeruli, are rarely reversible and potentially progressive [5]. A similar outcome of long-term Cs therapy was

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also considered in patients with autoimmune disorders receiving high Cs doses [3]. Cs effects on proximal tubular cells have been observed independently from a reduced GFR [1, 8], and might possibly be due to a generation of toxic metabolites by the tubular cell cytochrome P-450 system [1] or a direct effect of Cs on mitochondria of these cells [8].

Beside tubular, endothelial and vascular smooth muscle cells, glomerular mesangial cells (MC) exhibit several properties which could possibly link some of the functional and histological changes observed in the kidney to distinct cellular effects of Cs. As is also available for other kidney cell types, there are several recent investigations supporting MC involvement in Cs nephrotoxicity: Cs suppressed mesangial cell proliferation in vitro, while non-immunosuppressive Cs analogues were without effect [24]. Cs increased intracellular calcium and was responsible for an enhanced MC sensitivity to vasoconstrictors [9, 20–22]. Additionally, Cs induced platelet activating factor in MC, which in turn caused contraction of MC and whole glomeruli [10, 16]. Cs changed the pattern of prostanoid production towards vasoconstrictive compounds decreasing prostaglandin E_2 [17] and increasing thromboxane A_2 formation [1]. In vivo evidence supporting the pathophysiological role of the mesangial cell includes the generation of reactive oxygen species after Cs treatment [25], as MC are the main producers of reactive oxygen of the non-inflamed glomeruli [26]. Furthermore, MC proved to be more sensitive to Cs and its metabolites than murine lung and renal epithelial cells [27].

Cs metabolites are not easy to obtain, and to our knowledge only two in vitro studies have evaluated single Cs metabolite effects on tissue cells [20, 27]. On the other hand, an increasing number of investigators have described toxic Cs side effects associated with high metabolite levels [4, 6, 7]. Remarkably, in these studies a normal trough level of the parent drug Cs has been measured in the blood of patients.

Thus, the in vivo situation is complex and demands proper evaluation of the activities of metabolite combinations [6, 7, 28–30], especially as the only studies done so far with Cs metabolite combinations have shown synergistic immunosuppressive effects [28, 30]. In our Department 29 distinct metabolites have been detected in bile of liver grafted patients, and in addition to the 12 previously described metabolites the remaining 17 new metabolites have been structurally analyzed by FAB-MS [31]. Thus, patients have to deal not only with the presence of a few metabolites but with a combination of almost thirty Cs degradation products [28–32]. So far, nothing is known about the activities of secondary Cs metabolites on normal tissue cells and no information is available about the interactions of metabolite combinations.

In our initial experimental approach we have measured the inhibition of rat glomerular mesangial cell proliferation as a parameter for overall Cs metabolite activity. This proved to be a sensitive in vitro model system providing initial data, which might lead to more detailed studies of in vivo relevant endpoints of Cs metabolite actions like, for example, MC prostaglandin production or smooth muscle cell contractility. We have employed 10 HPLC-purified, ^{1}H NMR and/or FAB-MS characterized Cs metabolites from bile of liver graft recipients to determine the effects of single metabolites alone. Additionally, and more important for the in vivo situation, we have investigated the effects of metabolite combinations.

Methods

Materials

Cyclosporine (Cs) was a gift of Sandoz, Basle, Switzerland. The stock solution of 2 mg/ml was kept at −20°C in dimethylsulfoxide (DMSO). Reference Cs metabolites for the HPLC purification method (M1, M8, M13, M17, M18, M21, M26 and M203-218) were donated by Dr. G. Maurer (Sandoz). In a further collaboration with Dr. B. Ryffel, (Sandoz) we were supplied with chemically synthetized metabolites (M17, M18, M21, M203-218) in milligram amounts, which allowed us to perform control experiments under similar conditions as described below for the HPLC-purified metabolites prepared from human bile.

All cell culture reagents were from GIBCO (Wiesbaden, FRG), if not otherwise stated. The sources of the antisera for immunocytochemical characterization of MC were: myosin (rabbit anti-chicken) from R.B. Sterzel (Erlangen, FRG); fibronectin (MoAb anti-human), vimentin (MoAb anti-human), desmin (MoAb anti-human), and collagen type IV (MoAb anti-human) from ICN (Eschwege, FRG); cytokeratin (MoAb anti-cytokeratin-pan) from Boehringer (Mannheim, FRG); factor VIII (rabbit anti-human FITC conjugate) was a gift of Dr. F. Drenk (Dept. of Clinical Immunology, Medical School Hannover, FRG).

Preparation of cyclosporine metabolites

Cs metabolites were purified from human bile, which was obtained from liver grafted patients usually receiving 2 mg/kg Cs twice daily. For surgical reasons their bile was collected through a T-drain placed in the bile duct. The obtained bile was prepared according to our recently described extraction procedure [32] with the following modifications. In brief, 500 ml human bile were extracted by 500 ml dichloromethane. The dichloromethane phase containing cyclosporine and its metabolites was evaporated and the residue was dissolved in 150 ml acetonitrile/water pH 3.0 = 50/50 vol/vol. The sample was then cleaned by adding 300 ml hexane and was extracted from the aqueous phase by 150 ml dichloromethane. Dichloromethane was evaporated, the residue dissolved in 2 ml acetonitrile/water pH 3.0 = 50/50 vol/vol, cleaned by 4 ml hexane and 250 µl were injected into the HPLC system. The metabolites were eluted from preparative columns (3 serially linked 250 × 10 mm columns filled with 10 µm, 100 Å, RP8 material) by a concave gradient as described before [31, 32]. Fractions were manually collected and stored in acetonitrile/water (pH 3.0 = 50/50 vol/vol at 4°C).

Since the Consensus Conference in Hawk's Cay, USA, in June of 1990 [33], a new cyclosporine metabolite nomenclature has been introduced. However, for convenience we used the established nomenclature previously proposed by Maurer et al [34] and Christians et al [32] (indication of metabolites by the H-labeled numbers for metabolites as recently defined by our group). To permit a comparison with the new "Consensus Nomenclature," the following is a brief translation (old = new): M1 = M9; M8 = M19; M13 = M4N9; M17 = M1; M18 = M1c; M21 = M4N, M25 = M14N; M26 = M1c9; M203-218 = M1A. The new nomenclature describes metabolic alterations of the 11 amino acids of Cs by special suffices following the amino acid

number: no suffix = hydroxylation; N = N-desmethylation; c = cyclization; A = oxidation to an acid.

Mesangial cell culture

MC were prepared from Sprague-Dawley rat kidneys as described [24]. The cells were characterized by immunofluorescence showing a positive reaction with myosin, vimentin, fibronectin, desmin and collagen type IV, and negative reaction with keratin and factor VIII antisera.

The culture medium used for the experiments consisted of DMEM, supplemented with 2 mmol/liter glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 5% fetal calf serum (Gibco). MC cultures routinely proved to be negative for mycoplasma contamination by DAPI staining (4'6-diamino-2-phenylindole; Sigma, Deisenhofen, FRG).

Determination of cyclosporine metabolite activity

MC were seeded in 96-well flat bottom plates (Nunc, Wiesbaden, FRG) at a density of 5000 cells/well in culture medium and incubated in humidified atmosphere for 24 hours at 37°C in 5% CO_2. The HPLC purified, highly concentrated metabolites (300 to 600 μg of the respective metabolite per experimental series) dissolved in acetonitrile were dried in glass vials with nitrogen immediately before starting the assay. To increase the recovery the individual glass vials were washed three times with a respective volume of 5 μl DMSO (centrifugation at 12,000 g). Subsequently the vials were rinsed three times with 1.5 ml culture medium (5% fetal calf serum) to give the stock solutions for the serial dilutions. Similarly, the chemically synthesized metabolites for control experiments were dissolved first in DMSO and then in medium. The highest concentration of the metabolite dilutions applied to the MC contained not more than 0.16% DMSO. After 24 hours when MC were subconfluent either Cs, metabolites alone, metabolite combinations, medium or DMSO controls were added to a final volume of 200 μl/well. The cells were further incubated for 48 hours, and after adding 0.5 μCi [^{3}H]-thymidine for a further 24 hour period. The incubations were stopped by deep freezing, and then the thawed and sonified plates were harvested with an automatic cell harvester (Titertek). Radioactivity was determined by liquid scintillation counting.

The precise concentrations of the serial dilutions of Cs and Cs metabolites used were checked by HPLC in parallel to the respective incubation time. This became necessary as the recovery of purified (but also of chemically synthesized) metabolites in the final DMSO/medium solution varied considerably. The final concentrations of the metabolites shown throughout this paper were based on these parallel HPLC measurements.

To test the possibility that MC themselves were able to metabolize Cs we incubated MC in culture flasks (10^7 MC/flask) under identical medium conditions with 0.4 mg/liter native Cs for 72 hours. The incubation was stopped by adding acetonitrile to the separated supernatant and MC cell fraction. The samples were treated as described by Christians et al [32].

Determination of reversibility and cytotoxicity

The reversibility of Cs metabolite effects was determined by first incubating a duplicate set of MC with metabolites alone or in combination as described before. After 72 hours one-half of the metabolite treated MC was washed three times during a six hour period with culture medium containing 5% FCS, 37°C. Each washing step was preceded by a short centrifugation step of the microtiter plates (300 × g, 3 min) to avoid loss of cells. Subsequently, both the washed and the control cells, still exposed to the initial metabolite dilutions, were incubated for another 48 hours. Then both MC fractions were pulsed for 24 hours with [^{3}H]-thymidine and proliferation was determined as described before.

For cytotoxicity assays MC at a density of 5×10^4 cells/well were grown in culture medium in 96-well plates overnight. After addition of serial Cs metabolite dilutions in fresh medium and another 24 hours incubation time the microtiter plates were centrifuged (300 × g, 5 min) and the supernatant decanted. A 1/400 solution of neutral red dye in complete culture medium was added (150 μl/well) and living MC were allowed to incorporate the dye during an additional hour of incubation at 37°C, 5% CO_2. MC were washed twice with ice-cold PBS and cell incorporated neutral red dye was solubilized by the addition of 150 μl/well Sörensen-buffer and shaking of the microtiter plates. The neutral red absorption was determined at 540 nm in an ELISA reader.

Statistics

Each data point of Cs and single metabolite activities represents the mean of six determinations. In Figure 1 A and B the data points shown were raised in four (+ one for HPLC purified Cs) and three completely independent experiments, respectively: bile from different patients, separate HPLC extraction procedures, as well as separate performances of HPLC runs to assess the final metabolite concentrations. Concentration response curves calculated with the best curve fit are depicted. In all the remaining figures the data points represent the mean ± standard deviation (SD) of three independent experiments (6 determinations per experiment, respectively). For clarity the SD of the mean values in Figures 1, 2 and 3 have been ommitted. The mean standard deviation throughout this experimental series ($N > 500$) was 9.80 ± 4.47%.

Results

The metabolite concentrations depicted throughout the following figures refer to the actual concentrations measured by HPLC in the dilution medium. HPLC assessment in parallel to the respective experiment reconfirmed the purity of the human bile-derived metabolites and excluded alterations caused by DMSO or culture medium (data not shown). Moreover, control experiments with newly synthesized metabolites (M17, M18, M21, M203-218) were performed in addition to the studies on purified metabolites. The results revealed an identical activity of these synthesized preparations either alone or in combination, provided their respective concentration in the serial dilutions was measured by HPLC in parallel to the MC proliferation assay (data not shown). DMSO controls (0.16%, vol/vol) corresponding to the highest concentration reached with metabolites alone or with combinations caused a growth reduction by 6.64 ± 2.03 % (mean ± SD, $N > 100$) compared to medium controls.

In additional, preceeding experiments MC cultured in flasks were incubated with a concentration of 0.4 mg/liter native Cs for 72 hours. We determined that MC under these assay

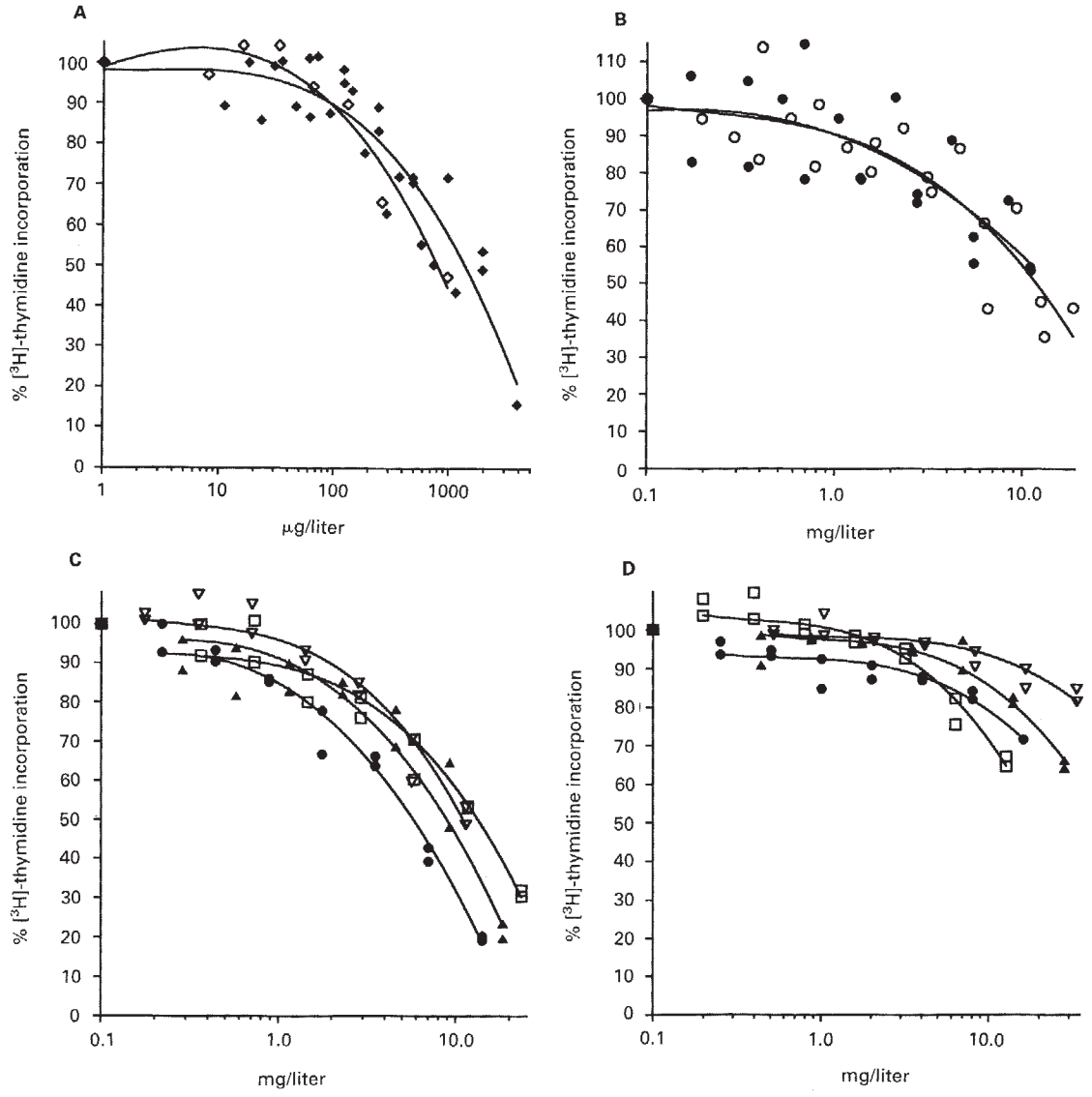

Fig. 1. *Antiproliferative effects of Cs and single metabolites.* The ordinate gives the percent of proliferation of MC at the respective metabolite concentration compared with control. Best regression curve fits were calculated: **A.** MC were incubated for 72 hours with native Cs (◆) and HPLC-purified Cs (◇) from human bile. Data points depicted represent the mean of six determinations. **B.** The activities of the metabolites M1 (○) and M8 (●) were determined in three independent experiments: bile from different patients, independent HPLC-purification procedures and HPLC-reassessments of the actual concentrations and different MC incubations. **C.** Antiproliferative effects of the metabolites M17 (□), M18 (▲), M21 (●), M26 (▽). Data points represent the average of three independent assays with six determinations, respectively. **D.** The activity of the secondary metabolites M13 (□), M25 (▲), M203-218 (●), H355 (▽) was determined as described in C.

conditions were not able to generate Cs metabolites (data not shown). The effect of Cs and metabolites on mesangial cell [^{3}H]-thymidine incorporation was dependent on cell density, that is, confluent, contact-inhibited MC were less sensitive (data not shown). Nevertheless, the relative activity of the metabolites was retained.

Effect of single cyclosporine metabolites

Native Cs caused a significant, concentration dependent decrease of MC proliferation starting at 0.1 mg/liter with a 50% antiproliferative action (IC_{50}) at a dose of 1.25 mg/liter (Fig. 1A, Table 1). Cs purified from human bile extracted as described resulted in a comparable growth inhibition curve.

Testing the metabolite activity revealed two separate groups of metabolites, the "active" metabolites M1, M8, M17, M18, M21, M26, (Fig. 1B and C) and the relatively "inactive", secondary metabolites M13, M25, M203-218, H355 (Fig. 1D). Figure 1B shows the concentration dependent effects of metabolites M1 and M8 from three independent experiments. Representative for this study, these data confirmed that the antiproliferative effects of the metabolites were not significantly influenced by bile from patients, metabolite preparation procedure, MC cultivation or by HPLC re-assessments, because all of these parameters were different in these three experiments. Figure 1C summarizes the activities of additional four active metabolites M17, M18, M21, M26, revealing that metabolite

Table 1. Summary of metabolite concentrations (mg/liter) resulting in 50% inhibition of proliferation (IC_{50}) of Sprague-Dawley rat mesangial cells compared with the chemical structure of the metabolites

		Structure			
	IC_{50}	AA9 R	AA1 R_1	AA3 R_2	Other modifications
Cyclosporine	1.25	H	CH_3	CH_3	
Active metabolites					
21 (H430), 1	6.0	H	CH_3	H	
18 (H400), 1	9.0	H	CH_2 OH	CH_3	AA1 cyclizat.
26 (H270), 2	10.5	OH	CH_2 OH	CH_3	AA1 cyclizat.
1 (H390), 1	10.8	OH	CH_3	CH_3	
8 (H250), 2	10.8	OH	CH_2 OH	CH_3	
17 (H370), 1	12.5	H	CH_2 OH	CH_3	
Inactive metabolites					
13 (H320), 2	>20.0	hydroxylation/N-demethylation			
25 (H300), 2	>25.0	H	CH_2 OH	H	
203-218 (H350), 2	>50.0	H	COOH	CH_3	
(H355), 2	>50.0	hydroxylated (not at AA9) M17			

For assay conditions see **Methods**.

The amino acid (AA) residues (R) are numbered as shown in Figure 7. The nomenclature of the metabolites proposed by Maurer et al [34] and in brackets according to Christians et al [32] was used. Identification as primary (1) or secondary (2) metabolites refers to studies of the metabolization pathways of Cs (Fig. 7) by Sewing et al [31].

M21 had the highest antiproliferative potency on rat MC with an IC_{50} of 6.0 mg/liter. The secondary metabolites M13, M25, M203-218 and H355 had an $IC_{50} > 20$ or 25 mg/liter as shown in Figure 1D. The IC_{50}'s of CS and the 10 metabolites tested on rat MC are summarized and compared to the their chemical structure in Table 1.

Effect of metabolite combinations

To get informations concerning the interaction of Cs metabolites on rat MC we tested the growth inhibitory action of several combinations of two, three or four metabolites. Figure 2 demonstrates the concentration dependent effects of the metabolite combinations M1 + M17 (Fig. 2A) and M203-218 + H355 (Fig. 2C), respectively. Whereas the combination of metabolites M1 + M17 produced an additive effect summarizing the growth inhibition of each metabolite alone (Fig. 2A), combination of the secondary metabolites M203-218 + H355 resulted in a significant synergism (Fig. 2C).

Combinations of three metabolites, triple combinations (Fig. 2), resulted in an additive antiproliferative effect of the metabolites M1 + M17 + M18 (Fig. 2B), but when metabolites M203-218, H355 and M21 were combined we found a synergistic activity (Fig. 2D). In the experiments employing the secondary metabolites M203-218 and H355 the synergism of both the double (Fig. 2C) and the triple combination (Fig. 2D) was more pronounced at low metabolite concentrations. The phenomenon that a combination of active metabolites reached not more than additive antiproliferative effects, whereas combined inactive metabolites led to synergism, was also observed in further experiments using the double metabolite combination M1 + M18 (additive) or the triple metabolite combinations M1 + M8 + M17 (additive) and M13 + M203-218 + H355 (synergistic) (data not shown).

A combination of four different metabolites (Fig. 2E) caused a remarkable growth inhibition of MC already at low concentrations of the participating metabolites. The antiproliferative effect of this quadruple combination was additive for the concentration range tested.

Influence of native cyclosporine on metabolite combinations

To test the antiproliferative effect of Cs together with metabolite combinations on rat MC we used a concentration of 0.625 mg/liter Cs. Cs alone at this concentration produced a growth inhibition of 30% (Fig. 3), which was expected from the concentration response curves (Fig. 1). The combination of the metabolites M1 + M17 + M18 (Fig. 3A, upper curve) or the metabolites M21 + M203-218 + H355 (Fig. 3B, upper curve) resulted in the described additive or slightly synergistic antiproliferative activities, respectively. Combining native Cs (0.625 mg/liter) with the triple metabolite mixture did not significantly enhance the growth inhibition in response to either Cs or the metabolite combinations (Fig. 3 A and B, lower curves). The effects seemed to be independent from each other.

Reversibility of Cs metabolite effects

To exclude the possibility of direct and nonspecific toxic effects of purified or synthesized Cs metabolites, we tested both the reversibility of the antiproliferative metabolite action after 72 hours and the effects of Cs metabolites on MC viability after 24 hours measured by neutral red staining. Figure 4 demonstrates that a washing procedure with FCS-containing medium after 72 hours incubation with single metabolites (Fig. 4A: M17; B: M18; C: M21 and D: M203-218) and a subsequent addition of fresh medium led to a full recovery of MC proliferation rate compared to controls. As an exception the effect of the highly lipophilic M21 (Fig. 4C) at the maximal concentration of 30.2 mg/liter was only partially reversed. Similarly, the growth rate of MC treated with triple (Fig. 5A: M17 + M21 + M203-218) or with quadruple combinations (Fig. 5B: M17 + M18 + M21 + M203-218) returned to control levels after 72 hours of metabolite-free incubation. Providing further evidence for an antiproliferative activity rather than a direct cytotoxicity of Cs metabolites, Figure 6 demonstrates the results of a living cell stain with neutral red dye of MC incubated for 24 hours with increasing concentrations of Cs metabolites. Neither single metabolites (Fig. 6A) nor triple and quadruple combinations

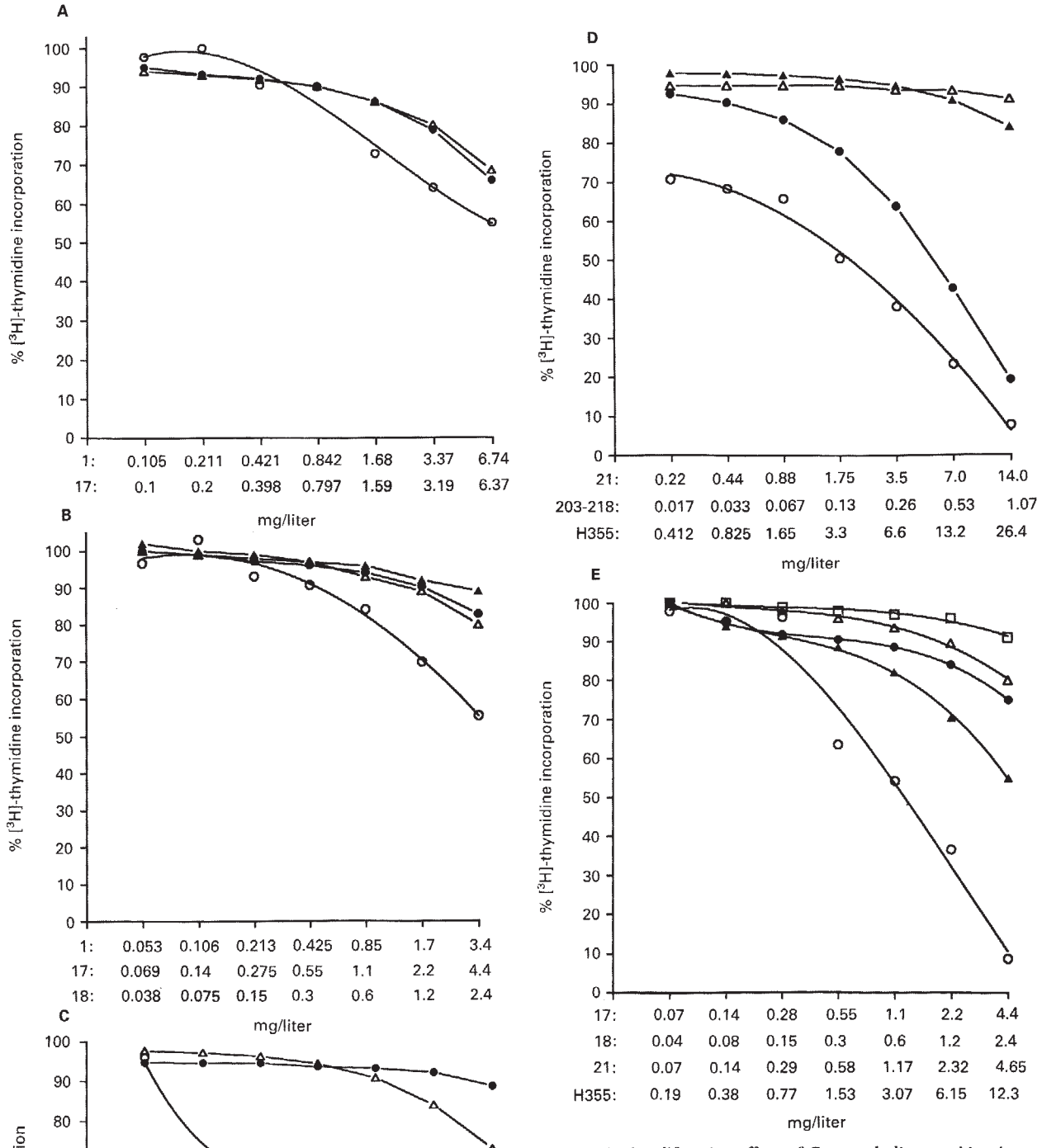

Fig. 2. *Antiproliferative effect of Cs metabolite combinations on MC.* The upper regression curves of each of the following figures refer to the growth inhibition obtained with single metabolites. The X-axis shows the concentrations of the respective metabolites contributing to the combined effect and/or when given alone. Data points for the combinations connected by best fitting regression curves represent the mean of 12 determinations of two experiments. **A.** A combination of the metabolites M1 + M17 (○) was added to subconfluent MC for 72 hours and compared with the activity of the metabolites alone (●, M1; △, M17). **B.** Antiproliferative effect of a combination of the metabolites M1 + M17 + M18 (○) or individually: (●) M1, (△) M17; (▲) M18. **C.** Synergistic activity of secondary metabolites M203-218 + H355 (○), which exhibited a very low activity when given alone (upper curves, ●, M203-218; △ H355). **D.** Synergistic growth inhibition of MC during the 72 hour incubation time by a combination of the primary metabolite M21 with the secondary metabolites M203-218 and H355 (○) or individually: (●) M21; (△) M203-218; (▲) H355. **E.** Antiproliferative activity of a combination of four metabolites: M17 + M18 + M21 + H355 (○), or individually: (●) M17, (△) M18; (▲) M21; (□) H355.

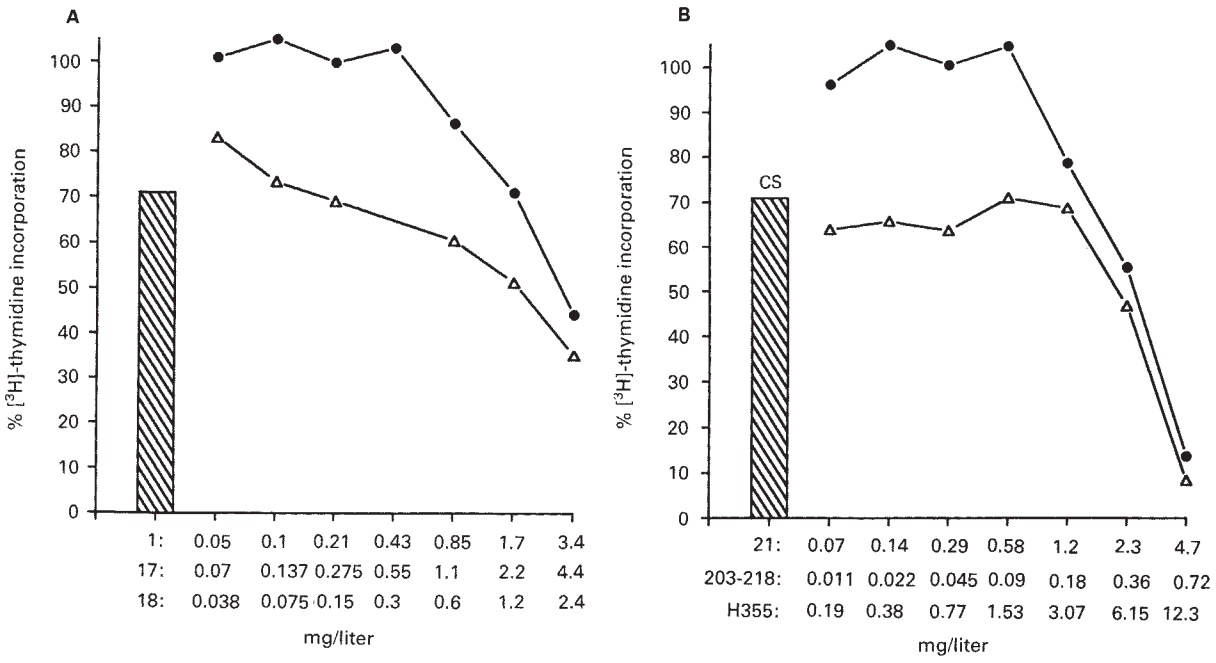

Fig. 3. *Combinations of three metabolites with (△) or without (●) Cs.* The antiproliferative effects of the combined metabolites M1 + M17 + M18 (**A**) and M21 + M203-218 + H355 (**B**) were tested on MC during a 72 hour incubation period without or with the addition of Cs at a concentration of 0.625 mg/liter. The results are the mean of six determinations (SD below 10%) of a representative experiment.

(Fig. 6B) caused a direct mesangiolysis. As a control we determined that even an excessively high concentration of the parent drug cyclosporine of 50 mg/liter only led to a lysis of 33.8 ± 2.9% of MC under these conditions.

Discussion

The aim of this investigation was to determine potentially toxic effects of a representative group of Cs metabolites from human sources in an in vitro system. We could demonstrate that combinations of Cs metabolites had clearly additive growth inhibitory effects on rat glomerular MC. Moreover, combinations of inactive metabolites acted synergistically.

As previously outlined measuring MC [^{3}H]-thymidine incorporation has to be taken as a simple, initial experimental setting to get informations about Cs metabolite effects on a renal, smooth muscle-like cell type. Using subconfluent MC being in the logarithmic proliferation phase provided us with a sensitive system to test Cs metabolite activities in a reasonable time. Control experiments clearly distinguished these antiproliferative effects of both single metabolites and metabolite combinations from unspecific mesangiolysis. The complete reversibility and the lack of any direct lytic effect together with the chemical structure related activities of single metabolites, discussed in detail later, point towards a specific action of the individual Cs metabolites and their combinations on rat MC. In this respect the model system used might provide useful informations for the analysis of other relevant target cell systems and their products.

The basis of our investigations with metabolite combinations was the evaluation of single metabolite effects. We used a standardized HPLC-procedure of metabolite purification and characterization from human bile [discussed in 31, 32], which permitted generation of sufficient material for the experimental series. Control experiments with small amounts of synthesized metabolites (M17, M18, M21, M203-218) revealed identical results and confirmed the reliability of the metabolite preparation method using HPLC (data not shown). The activities of metabolites either purified or synthesized were dependent on the degree of metabolization when tested alone (Fig. 7, Table 1). The rank order is Cs ≫ M21, M18, M26, M1, M8, M17 ≫ M13, M25, M203-218, H355. The group of active metabolites included four primary metabolites (M1, M17, M18, M21), which are either hydroxylated or demethylated products of cytochrome P450-dependent liver metabolization (Table 1). It is noteworthy that metabolite M21, which according to its HPLC elution time appeared to be closest in the lipophilic nature to Cs, exhibited the highest activity in our assay system. Additionally, two secondary metabolites (M8, M26) showed an IC_{50} comparable with that of the primary metabolites. Both of the latter metabolites have a chemical structure closely related to primary metabolites, especially with respect to the biologically important structure at amino acid residue 1 (AA1) [9]. M8 and M26 share an additional hydroxylation at AA9. It is therefore tempting to speculate that modifications at this part of the Cs molecule have no biological significance [29]. The group of the secondary metabolites (M13, M25, M203-218, H355) was tested for the first time in this investigation. These compounds were generated in vivo from primary metabolites by a further demethylation (M13, M25), oxidation (M203-218) or hydroxylation (H355) (Table 1). Taken together there seemed to be an inverse relationship between structural changes along the metabolic pathways (Fig. 7) to more hydrophilic and polar compounds and the antiproliferative activities on rat MC. The concentrations we measured for single metabolites antiproliferative activities were in agreement with recent in vitro studies [24, 27] and we would not expect nephrotoxic effects of single metabolites (35) as the necessary high concentrations have not been observed in vivo (36, Table 2).

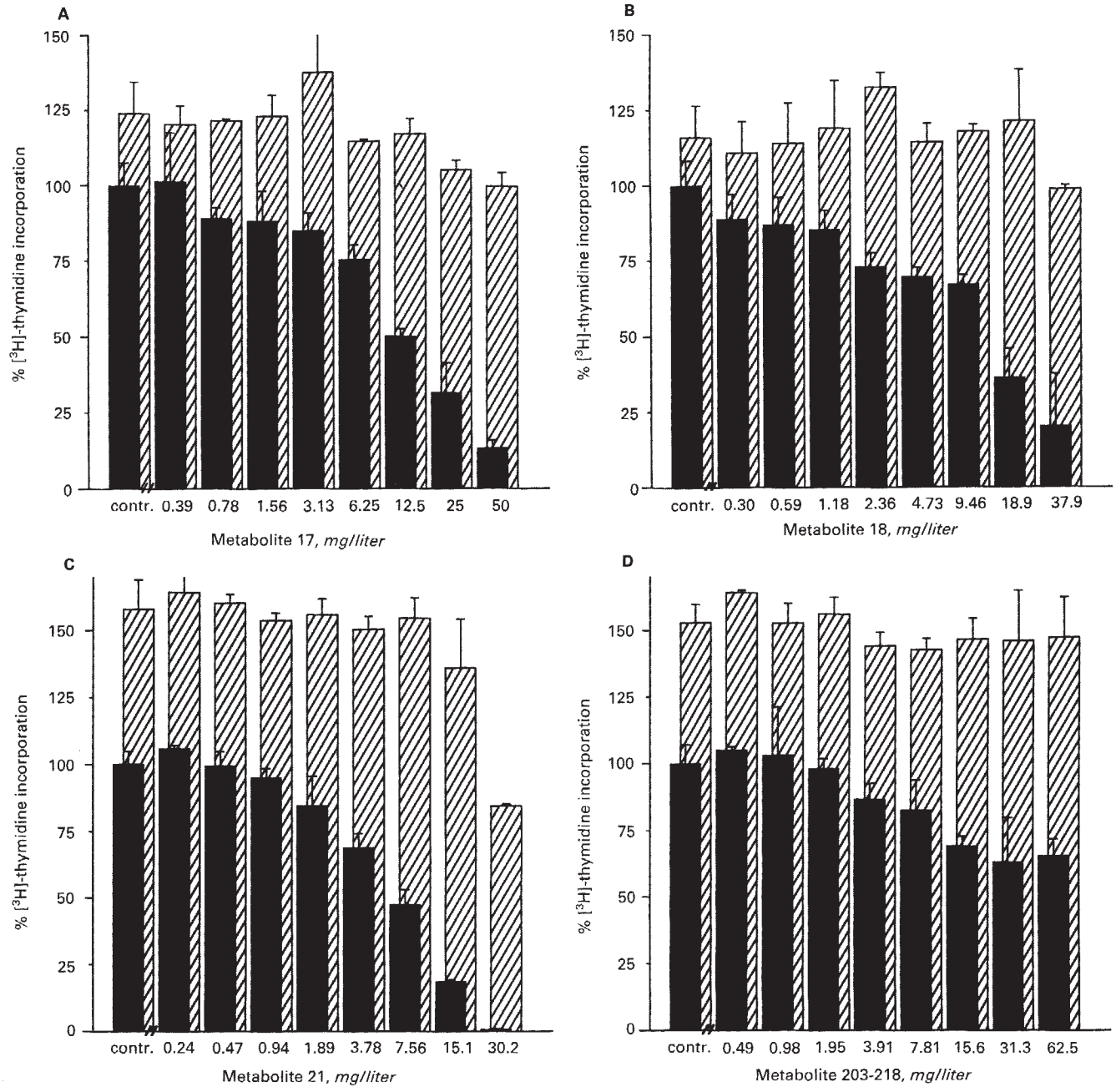

Fig. 4. *Reversibility of single cyclosporine metabolite effects.* Two parallel sets of subconfluent rat MC were incubated with serial dilutions of single metabolites for 72 hours as described in Figure 1. One part of the samples was incubated without removing Cs metabolites for another 72 hour period (■). The second identical set was washed three times with 5% FCS containing medium after the initial 72 hours, supplemented with fresh metabolite-free medium and incubated for additional 72 hours (▨). Except for M21 at 30.2 mg/liter (**C**) the proliferation rate of washed MC returned back to control levels for all concentrations and metabolites tested (**A**: M17; **B**: M18; **C**: M21; **D**: M203-218). Results expressed as percentage of control proliferation are the mean ± SD of three separate experiments performed with sixfold determinations.

The second part of our experimental series was to investigate the activities of metabolite combinations. As outlined previously, Cs treated patients have to deal with the presence of 29 different metabolites. In a first attempt to get closer to this in vivo situation we tested different combinations of two, three (± native Cs) or four metabolites. The only published investigation using well defined metabolites, in which at least a combination of two compounds was tested (Cs + M17), described a synergistic immunosuppressive effect [30]. In addition, in a recent paper Wallemacq et al [28], also testing the immunosuppressive activities of HPLC-purified metabolites, have demonstrated a "possible synergistic" action of unidentified metabolites from a more polar peak. In our own investigation on the immunosuppressive activities of Cs metabolites in a parallel experimental

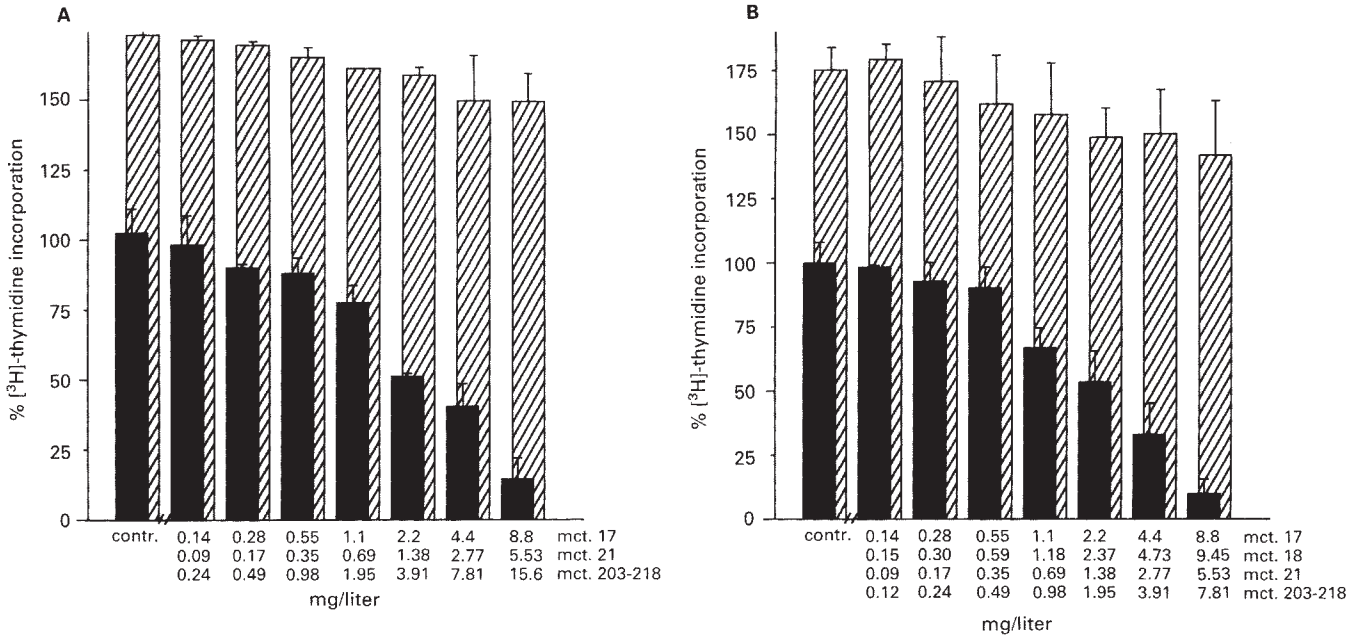

Fig. 5. *Reversibility of the MC growth inhibitory effects of Cs metabolite combinations.* As described for single metabolites (Fig. 4) three washing cycles with 5% FCS containing medium after an initial incubation period of 72 hours led to a complete reversibility of the concentration dependent inhibition of MC growth by triple **(A)** or quadruple combinations **(B)** after an additional 72 hour incubation time. Symbols are: (■) ^{3}H-thymidine incorporation in the presence of triple or quadruple combinations; (▨) ^{3}H-thymidine incorporation after three washing cycles. The means ± SD of three experiments with sixfold determinations are depicted as percentage of control.

series with Sprague-Dawley lymphocytes (Radeke et al, manuscript in preparation) we also found slightly synergistic activities of Cs in combination with metabolite M17 or with metabolite M203-218. However, and this refers to the results presented herein, the most prominent synergistic effects were observed, when we used secondary metabolites like M203-218 and H355 in combination. On rat MC these compounds generated in a second cycle of liver metabolization (Fig. 7), although exhibiting very low activity when given alone, acted synergistically or enhanced the activity of additionally present primary metabolites (metabolite M21).

Combinations from the group of active metabolites led to additive effects. But, obviously this might not be less important in vivo, since the concentration of the individual metabolites, which participated in the strong antiproliferative effects (for example in the quadruple combination), were in the range of metabolite concentrations measured in vivo [34] (Table 2).

Table 2 summarizes trough blood and urine metabolite levels of three different groups of Cs treated patients. These data reveal that the urine concentrations exceeded the blood trough levels and that the ratio of urine to blood concentration further increased with enhanced impairment of liver function. Supporting these measurements from our own laboratory, Rosano et al measured much higher tissue concentrations of Cs, metabolite M1 and M17, especially in the renal cortex compared to blood concentrations [36]. If we sum up the effects of the urine concentrations of the metabolites (without native Cs effect) occurring in group III (Table 2, liver allograft patients) the results in our in vitro system would be a 50% inhibition of MC growth. It has to be mentioned that the concentrations measured in patients' blood and urine represent trough levels and these are the baseline levels occurring during Cs therapy. In addition only ten metabolites would be sufficient to cause a 50% reduction of MC proliferation, whereas thirty metabolites are present in the vicinity of human MC in vivo. Ryffel [37] showed that Sprague-Dawley is the most insensitive rat strain, which increases the evidence that by analogy these concentrations might also be relevant for human glomerular MC.

While native Cs alone was relatively active on MC, we observed no significant additive action of Cs in combination with different metabolites. The reasons for this discrepancy compared with the combined metabolite activities are not known. In mesangial cells Cs has been shown to act on the cell membrane level to increase the availability of intracellular calcium. This leads to increased responses to vasoconstrictors [20, 21]. On the other hand, the nuclear actions of Cs on transcription regulator genes are known to be most important for the immunosuppressive activity [1, 38]. However, Fahr, Hiestand and Ryffel [29] have recently examined the immunological effects of Cs and several metabolites in comparison with their binding affinity to cyclophilin. Interestingly, although both M17 and M21 had a comparable high activity at inhibiting interleukin 2 mRNA expression of human lymphocytes and at suppressing mixed lymphocyte reaction in a mouse system, only M17 showed relevant binding affinity to cyclophilin. Thus, even in typical target cells for Cs metabolite actions there was no clear association at this level which suggested different pathways leading to the final effect. Similarly in MC different cellular target systems might exist for either Cs and/or its

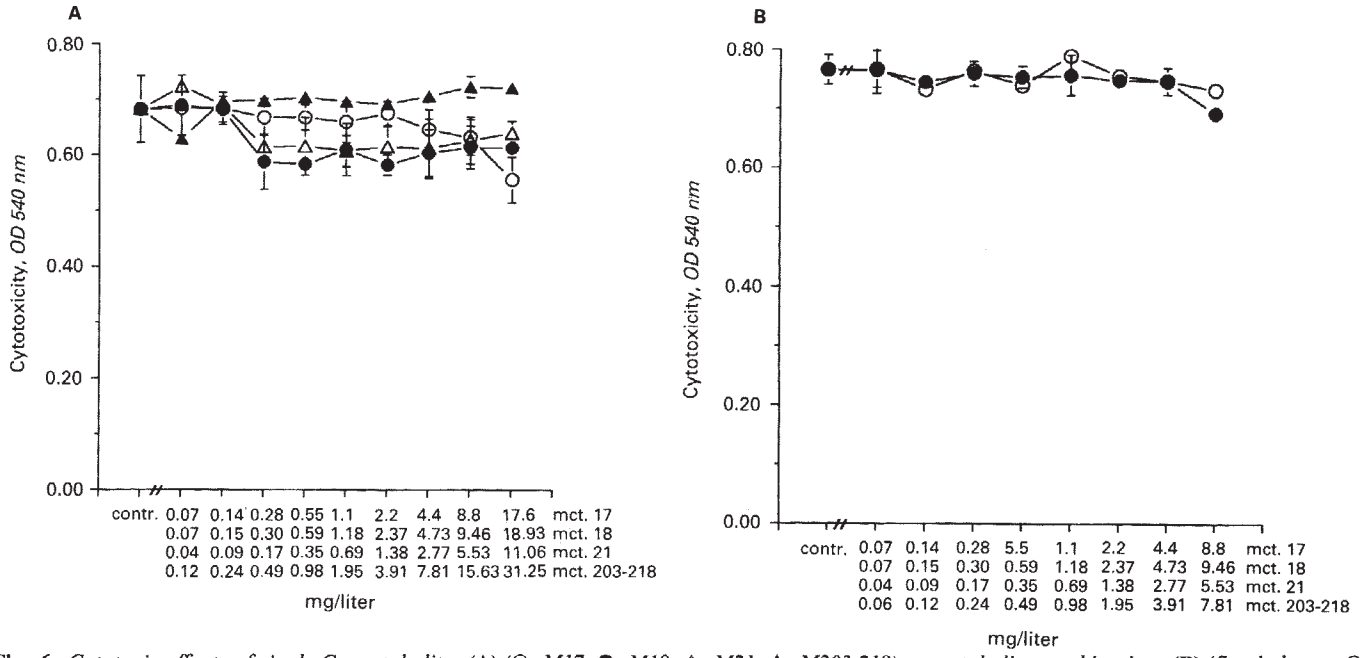

Fig. 6. *Cytotoxic effects of single Cs metabolites* **(A)** *(○, M17; ●, M18; △, M21; ▲, M203-218) or metabolite combinations* **(B)** *(Symbols are: ○, combination M17 + M21 + M203-218; ●, combination M17 + M18 + M21 + M203-218) on rat MC.* Five times 10^4 confluent MC/well in microtiter plates were incubated with the respective metabolite concentrations for 24 hours, washed and viable MC stained with neutral red dye for one hour. The absorption at 540 nm was determined in an ELISA-reader after the solubilization of the dye with Sörensen-buffer. The results are derived from one representative out of a series of three comparable experiments and expressed as mean ± SD of triplicates.

Table 2. Cs and metabolite trough levels (μg/liter, mean ± SEM) in blood and urine of kidney and liver allograft patients

	Group I		Group II		Group III	
Metabolites	Blood	Urine	Blood	Urine	Blood	Urine
Cyclosporine	135 ± 9	329 ± 41	159 ± 19	406 ± 74	175 ± 26	467 ± 105
21 (H430)	5 ± 4	85 ± 13	34 ± 19	81 ± 16	5 ± 4	85 ± 13
18 (H400)	10 ± 5	412 ± 74	37 ± 31	891 ± 451	14 ± 5	587 ± 130
26 (H270)	1 ± 1	255 ± 51	10 ± 8	736 ± 340	18 ± 6	1,490 ± 308
1 (H390)	73 ± 7	262 ± 32	159 ± 81	497 ± 205	53 ± 14	593 ± 116
8 (H250)	32 ± 4	162 ± 36	123 ± 52	616 ± 275	152 ± 29	2,260 ± 522
17 (H370)	180 ± 12	877 ± 106	232 ± 38	1,464 ± 434	157 ± 27	1,668 ± 327
13 (H320)	4 ± 2	48 ± 10	—	115 ± 42	21 ± 6	689 ± 14
25 (H300)	3 ± 2	32 ± 11	—	118 ± 70	3 ± 2	582 ± 141
203-218 (H350)	12 ± 4	29 ± 7	40 ± 13	228 ± 94	22 ± 6	1,378 ± 306
(H355)	—	4 ± 2	—	21 ± 9	—	4 ± 2

Data were adapted from an investigation of BLECK JS, SCHLITT HJ, CHRISTIANS U, SCHOTTMANN R, THIESEMANN C, STROHMEYER S, KOHLHAW K, WONIGEIT K, PICHLMAYR R, SEWING K-F: Kidney and liver function dependent change of cyclosporine metabolite pattern in blood and urine in kidney grafted patients (manuscript in press) and [32], respectively, and measured with the equipment described in the method section. Nomenclature of the metabolites is described in Table 1. Group I consisted of 19 kidney graft patients with normal liver function, group II of 8 kidney graft patients with cholestasis and group III of 18 liver graft patients. None of the patients received additional medication known to interfere with metabolization and/or elimination of the daily cyclosporine dose of 2.0 ± 1.3 mg/kg body wt or 2.9 ± 1.2 mg/kg body wt of the kidney or liver graft patients, respectively.

metabolites. Nevertheless, in our experiments the combinations of metabolites still had antiproliferative effects on MC in the presence of Cs. These in vitro results are in agreement with observations in patients who suffered from severe complications which were independent of Cs trough levels [4, 6, 34].

Finally, we conclude that: 1) concentration dependent effects of single metabolites on rat MC were observed. The activities were correlated to the degree of metabolization. 2) The combined addition of two, three or four metabolites to target cells resulted in additional antiproliferative effects of the active metabolites (M1, M8, M17, M18, M21, M26). This might be relevant in vivo, since the concentrations of the respective metabolites contributing to the overall effect were measured in urine of Cs treated patients. 3) Moreover, secondary metabolites with a higher degree of metabolic alteration, although exhibiting low activity when given alone, showed synergistic growth inhibiting effects on rat MC. Chronic toxicity of Cs therapy is a major problem and more detailed studies using metabolite combinations may help to develop strategies to decrease undesired effects.

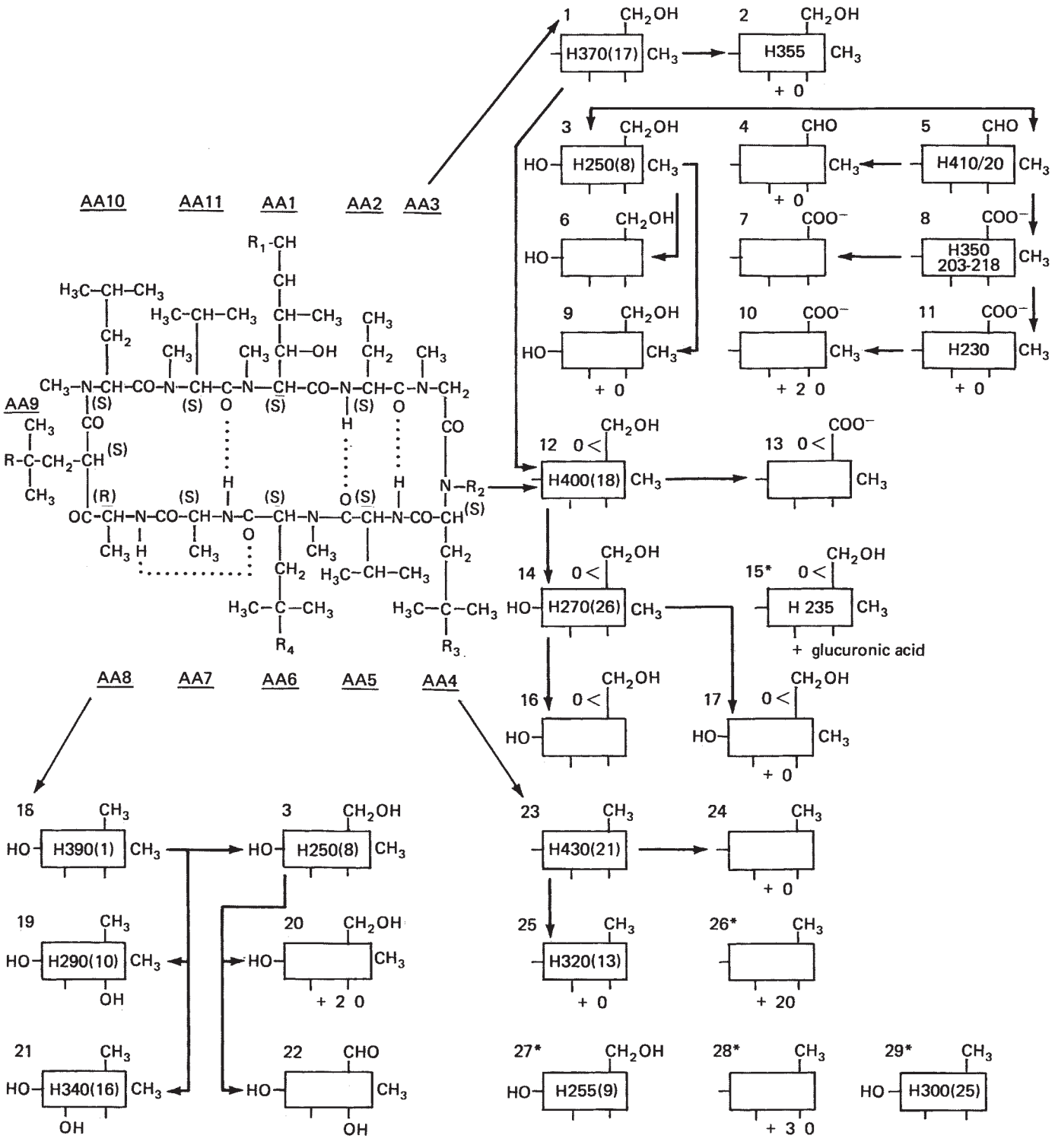

Fig. 7. *Metabolization pathway of cyclosporine adopted from [31].* The cyclosporine formula is skeleted to the cyclic structure and its metabolization sites. The numbers in the metabolite formulas correspond to the numbers in Tables 1 and 2, and use the nomenclature of Maurer et al [34] and Christians et al [32].

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